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# Surface Plasmon Resonance Biosensors for Highly Sensitive Detection of Small Biomolecules

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## 1. Introduction

Small biomolecules ( $< 2$  kDa) are crucial to a wide range of biochemical processes in the human body. Their diverse functions can include acting as hormones, neurotransmitters and pheromones. Small molecules are typically found in the blood stream and their concentrations can often be indicative of the biochemical functioning of the organism. This is particularly true of the steroids, a major class of small molecule hormone.

Measurement of steroid concentrations is commonly performed to track the progression of the fertile cycle, to detect pregnancy and to help diagnose hormonal diseases (Clark et al., 1998). Immunoassay techniques dominate the market for hormonal assays. These assay kits utilise the highly selective binding of an antibody to its target antigen to detect small molecule analytes in highly complex matrices. Typically, for small molecules, the assay will consist of a plate or tube onto which the antibody is immobilised and the sample is passed over the plate surface along with an aliquot of labelled antigen. The label used is either radiochemical (radioimmunoassay (RIA)) or enzyme-based (enzyme-linked immunosorbent assay (ELISA)). The labelled antigen occupies the un-bound sites on the immunoassay plate and the signal obtained from the label by either measuring the resultant radiation, or through enzyme catalyzed colour changes, gives a standard curve from which the concentration of the free analyte can be determined.

Small molecule targets are typically found in complex matrices such as blood and saliva where high molecular mass components predominate. These include proteins that can in some cases bind to the steroid hormone of interest. Their large size relative to the target antigen can also serve to sterically impede antibody binding to the steroid. Furthermore, large molecule contaminants can also bind to immunoassay surfaces causing biofouling that can further interfere with assay results.

Despite their widespread application, RIA and ELISA are practically limited to use in centralised laboratory environments where careful control of the experimental conditions can be exercised. They typically involve multiple steps that require trained operators and in the case of RIA they involve the use of radioisotopic labels that require specialised handling and represent a significant health hazard. Analysis is also very labour intensive and consequently quite expensive. Samples therefore must be transported to a laboratory and

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testing and processing of results typically takes several hours-days. This arrangement is inadequate for a wide range of applications that need results in much shorter timeframes.

To help address these issues, there has been much research devoted to the development of immunochromatographic test strip technology (Weller, 2000). This technology has been utilised for detection of drug residues (Chen et al., 2009) and drugs of abuse by transferring the immunoassay process onto a single-use test strip that generates a colour response after the sample has flowed over the immobilised protein conjugate. Such tests however, are largely semi-quantitative and so can be of limited use when a concentration result is required rather than a simple yes/no answer.

Biosensors offer a very promising means of achieving more versatile determination of small molecule concentrations in diverse matrices. A biosensor is an instrument that utilises a biomolecule/s, such as an enzyme or antibody, to interact with the target molecule being measured. From this interaction an electrical signal is then generated which can be used to determine the concentration of the target compound. Biosensors are typically classified according to the biochemical interaction used and the method of transduction. For detection of small molecules, antibody based biosensors are a logical choice as they retain the excellent specificity of antibodies allowing for detection in complex matrices, including those with a number of structurally similar compounds present in the same sample, as is the case with the steroid hormones. Furthermore, their relatively high binding affinities allow efficient uptake of the analyte from the sample and thus maximise the number of binding events and the biosensor signal. High quality antibodies are widely available for a broad range of small molecule analytes, including most of the physiologically significant steroid hormones.

When we examined the range of transduction technologies available for small molecule biosensing, we decided to investigate the use of the quantum optical-electrical phenomenon known as surface plasmon resonance (SPR). When a photon of light is incident on a noble metal surface (typically gold or silver) it can couple with the electrons in the metal, exciting the electrons and causing them to move as a single electrical entity known as a plasmon which propagates parallel to the metal surface. As the plasmon oscillates it generates an electric field that extends about 300 nm out from the metal surface (Homola et al., 1999). At a particular angle where the energy transfer from the photon to the plasmon is well matched a resonance occurs, significantly increasing energy transfer from the photon to the plasmon and causing a corresponding decrease in reflectance. If mass is bound to the surface within this 300 nm range then it perturbs the plasmon and, for a photon of fixed wavelength, shifts the resonance angle. If the plane of the metal surface is fixed, then the resonance wavelength can be identified by scanning through a range of angles of incidence for the photon and determining the resonance angle (Mullet et al., 1998). An SPR biosensor effectively measures minute changes in refractive index corresponding to binding events on the sensor surface (Homola, 2008). SPR biosensors can typically achieve resolutions down to 1 resonance unit (RU) which is  $1 \times 10^{-6}$  refractive index units (Stenberg et al., 1991). SPR biosensors can effectively detect binding by molecules as small as about 2 kDa, but smaller molecules generate insufficient changes in bound mass and so cannot be directly measured adequately. This poses particular challenges for detecting small molecules. In this case it is necessary to detect the binding of an antibody to an analyte conjugated sensor surface or to tag the small molecule with a large molecular mass tag and use this to bind to un-bound antibody sites on the sensor surface. Once the binding event/s have taken place, then the sensing surface can be returned to its original state by application of a suitable regeneration cocktail, allowing some re-use of the surface.

BIAcore SPR instruments have been widely used for years for the analysis of biomolecular interactions. It is extensively applied as a research tool to examine antibody / antigen (McCormick et al., 2004; Kubitschko et al., 1997), oligonucleotide / complementary oligonucleotide (Kai et al., 1999), receptor / target (Zhang et al., 2004) and host / guest (Chen et al., 2002) interactions. These instruments contain sophisticated microfluidics and temperature control units and range from the conventional four channel versions such as BIAcore 3000, to the more recent array format in the BIAcore Flexchip. The instruments use chips which contain the gold sensing surface and can be docked and un-docked from the main instrument as required. The work discussed in this chapter uses either a BIAcore 2000 or 3000 in the 4-channel format with a carboxymethylated (CM) dextran coating.

SPR biosensor assays have the advantages that binding data can be collected in real-time as the sensorgram (plot of response vs. time) is generated and so there is no need to wait for the signal development of an entire immunoassay plate to obtain results. This makes the technology particularly amenable to use in applications where data is required to inform decisions in near-real-time, such as in the diagnosis of disease or in the study of human hormonal responses. Furthermore, SPR assays are semi-automated. Once the samples are loaded into an instrument such as a BIAcore, then a wizard program can run the assay automatically and feed results to your desktop. With pre-prepared standards very little pipetting is required and this significantly reduces costs. SPR assays also do not use any radioactive labels. Increasingly, SPR biosensor technology is improving to the extent that a number of more compact and affordable options are now appearing on the market thus beginning a wider dissemination of the technology beyond large, well-funded centralised laboratories.

Potentially SPR is a very sensitive transduction technique where only small changes in refractive index are required to generate signal. This means that provided the immunoassay format and surface coating chemistries are well-designed, very sensitive assays can be achieved. However, in the case of small molecule immunoassay, the lack of mass in the analyte means that specialised enhancement technologies need to be applied to achieve the very low detection limits needed for measurement at the physiologically relevant concentrations found in certain biological fluids, including saliva.

It is very easy for steric hindrance to occur between the small molecule and the large antibodies used or between the small molecule and the sensing surface. If the analyte is sterically impeded from binding to the antibody this reduces the sensor signals that can be obtained and worsens the sensitivity of the immunoassay. Given that an SPR sensor detects bound mass on the sensor surface, if large molecules bind non-specifically to the surface then they will generate an SPR signal which can obscure the signal results from specific immunoassay binding of the analyte. This risk is particularly high if the sample matrix contains high concentrations of high molecular mass species, such as in the case of saliva which is rich in mucins. For a given assay it is very beneficial to be able to use the same biosensor surface over and over again many times. This approach means that the need for calibrating different sensor surfaces is minimised, cost of consumables is reduced and risk of failure of the sensing surface during crucial analyses is mitigated. Most SPR sensor surfaces rely upon self-assembled monolayers (SAMs) to conjugate small molecules or antibodies to the sensor surface. This method of attachment lacks longer-term stability as do most direct immobilisations of proteins onto sensor surfaces. Typically such SAM surfaces only last about 100-400 binding and regeneration cycles (Yuan et al., 2007).

Given these significant challenges faced by small molecule SPR immunosensing it is very important to consider how such assays can be rationally designed to overcome problems of steric hindrance, biofouling, sensor surface stability and signal enhancement for greater sensitivity. In this chapter we examine the strategies taken in our laboratories to overcome these challenges and to help make SPR immunosensing a more practical technique for sensitive detection of small molecules, even in complex matrices.

## 2. Rational design of steroid conjugates

To optimise the sensitivity of SPR immunoassays of the steroid hormones, it is necessary to achieve maximum specific antibody binding to the antigen-conjugated sensor surface. The first step in ensuring this is to allow free access by the antibody to the specific parts of the steroid molecule that the antibody recognises, that is the epitopes. To do this one must conjugate the steroid through a position distant from these critical functional groups and not conjugate through an existing functional group. For the steroids, this can be done by conjugating to the A-ring 4-position using the formation of a thioether linkage (Fig. 1). This technique was selected after SPR analysis revealed much stronger binding to the 4-position for progesterone than was obtained through the more conventional 7-position (Wu et al., 2002) (Fig. 2).

In the case of estradiol, another conjugation technique utilising a Mannich reaction to conjugate at the 2-position of the A-ring was found to be equally useful in retaining strong binding responses. This was in contrast to hemisuccinate conjugation through the hydroxyl group at the 3-position, which gave only 2% as much binding (Mitchell et al., 2006).

The next factor to consider is the length of the intermediate linker that spaces the antigen from the sensing surface (Bieniarz et al., 1996). The linker should project the antigen into the flow of fluid that passes through the biosensor flow cell and so allow optimal presentation of the immobilized antigen to the antibody thus ensuring maximum sensor signal. We have considered the effects of incremental changes in linker length for progesterone – protein conjugates, examining linkers of 4-, 11- and 18-atoms in length (Wu et al., 2002). The linker length was found to be crucial to the amount of antibody binding in the SPR flow-through biosensor format with binding responses increasing up to 18-atoms in linker length (Wu et al., 2002). Based on these observations, an oligoethylene glycol linker was attached to the steroid at the 4-position through a thioether bridge. This produced a 19-atom linker chain (Mitchell et al., 2005). Oligoethylene glycol is well known as being non-immunogenic

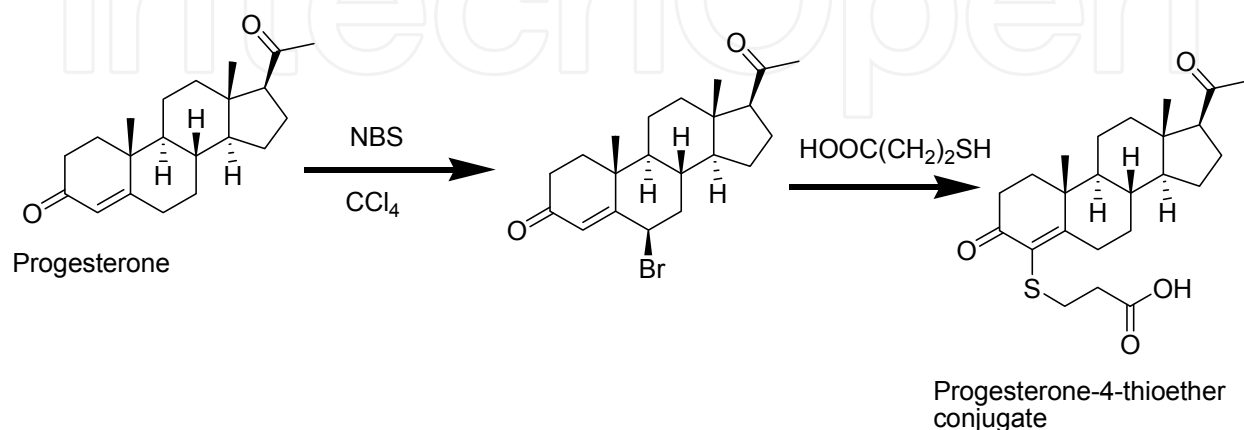


Fig. 1. Synthesis of progesterone-4-thioether conjugate for attachment of linkers



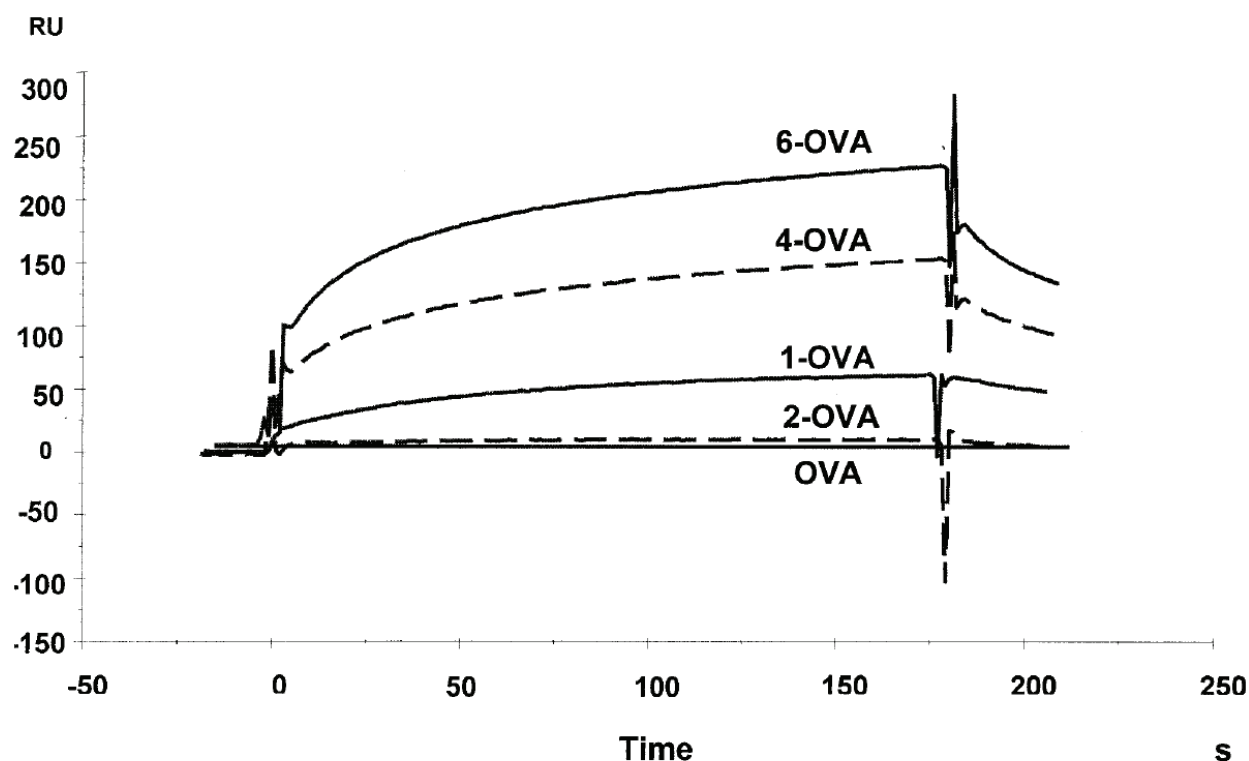


Fig. 2. SPR sensorgrams showing the binding of progesterone – protein conjugates to a progesterone monoclonal antibody (mAb) functionalised surface. OVA: ovalbumin, 2-OVA: conjugate through the 7-position, 1-OVA: conjugate through the 4-position, 4-OVA: 4-position conjugate with 11-atom linker, 6-OVA: 4-position conjugate with 18-atom linker. Reprinted from Steroids, 67, Mitchell, J. S.; Wu, Y.; Cook, C. J.; Main, L. (2002) Evaluation of progesterone-ovalbumin conjugates with different length linkers in enzyme-linked immunosorbent assay and surface plasmon resonance-based immunoassay. 565-572, 2002, with permission from Elsevier.

(Kurusu et al., 2003) and biocompatible (Otsuka et al., 2003) thus minimising non-specific binding to the linker. It is hydrophilic and thus allows good projection of the antigen into the aqueous mobile phase. Furthermore, it has high chemical stability and the length can be incremented as required by addition of more ethylene glycol units. Attachment to one end of the chain is achieved by protecting the amine at the opposite end by attachment of a *t*-butoxy carbonyl group which can be conveniently removed at the time of immobilisation.

### 3. Covalent linker conjugation technology

#### 3.1 Immobilization

High immobilization stability can be achieved by use of a totally covalent immobilisation of the steroid antigen to the sensor surface. In these studies, the most effective means of achieving this was to utilize a carboxymethylated (CM) dextran polymer coating applied to the gold surface. This dextran hydrogel extends for about 100 nm from the gold surface and is highly porous, giving a very high surface area. The polymer is studded with carboxylic acid groups which can be conveniently used as anchor or immobilisation points for covalent attachment of steroid-linker conjugates. This attachment is achieved by the formation of an

amide linkage between the carboxylic acid groups and the primary amines at the end of the oligoethylene glycol linkers. This can be further facilitated by use of *N*-hydroxysuccinimide (NHS) activation of the carboxylic acids.

Immobilization was achieved by simply flowing a mixture of NHS and *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide (EDC) over the CM dextran surface to activate it and then flowing the steroid linker conjugate. This is ideally performed at low flow rates (5  $\mu\text{L}/\text{min}$ ) to ensure maximum conjugation to the sensor surface. This technique provides high binding capacity surfaces without the need to disassemble the chip. Finally, ethanolamine solution (pH 8) can be passed over the surfaces to cap any un-conjugated carboxylic acid groups and so prevent them from reacting with proteins in the samples.

### 3.2 Surface stability and regeneration

To achieve a high throughput of samples and standards over a single surface, it is necessary to have very high sensor surface stability as the surface must be able to withstand multiple binding and regeneration cycles. The regeneration process involves exposing the surface to harsh regeneration “cocktails” which chemically remove the bound antibodies. The cocktails used in this work are typically 50 mM NaOH with 20%v/v acetonitrile, providing a combination of high pH and a chaotropic reagent. Despite exposure to such a harsh mixture, progesterone conjugated surfaces are able to withstand more than 1100 binding and regeneration cycles without appreciable loss of binding capacity (Mitchell et al., 2005). This greatly extends the lifetime of a single sensor surface and allows extensive re-use without the need for immobilization of a fresh surface and in so doing, significantly reduces the costs per assay cycle.

## 4. Assay format and gold nanoparticle signal enhancement

The assay format adopted was a competitive inhibition assay. In this format, the small molecule analyte was covalently immobilised on the surface. The sample containing the small molecule was then mixed with a primary antibody raised against the analyte and the mixture injected over the sensor surface. Primary antibody that had not bound the “free” sample antigen, bound the surface immobilized antigen thus producing a refractive index change on the surface (Fig. 3). This SPR signal is inversely related to the concentration of the “free” antigen and a standard curve can be produced that takes the form of an S-curve.

The sensitivity of the assay can be assessed as the amount of signal change achieved per unit of analyte concentration change and the limit of detection as the minimum concentration that can be statistically distinguished from the blank. Lower concentrations of the analyte can be detected by reducing the concentration of the primary antibody as less free antigen would be required to inhibit antibody binding to the antigen-immobilized surface. The problem with this approach in SPR, is that this will also reduce the signal by reducing the bound mass on the surface. To achieve a highly sensitive assay, it is therefore crucial to minimise the primary antibody concentration whilst retaining high signal.

When the above assay design was applied to the SPR biosensor as described, it gave assays with a LOD of approximately 1 ng/mL in the case of progesterone. Whilst this LOD is suitable for measurement of steroids in human blood serum, it is far too high to measure steroids in other matrices such as saliva, where concentrations are typically 1% of those found in blood. To shift the assay curve to lower concentrations, it was necessary to further reduce the primary antibody concentration. To retain sensor signal, strategies for labelling

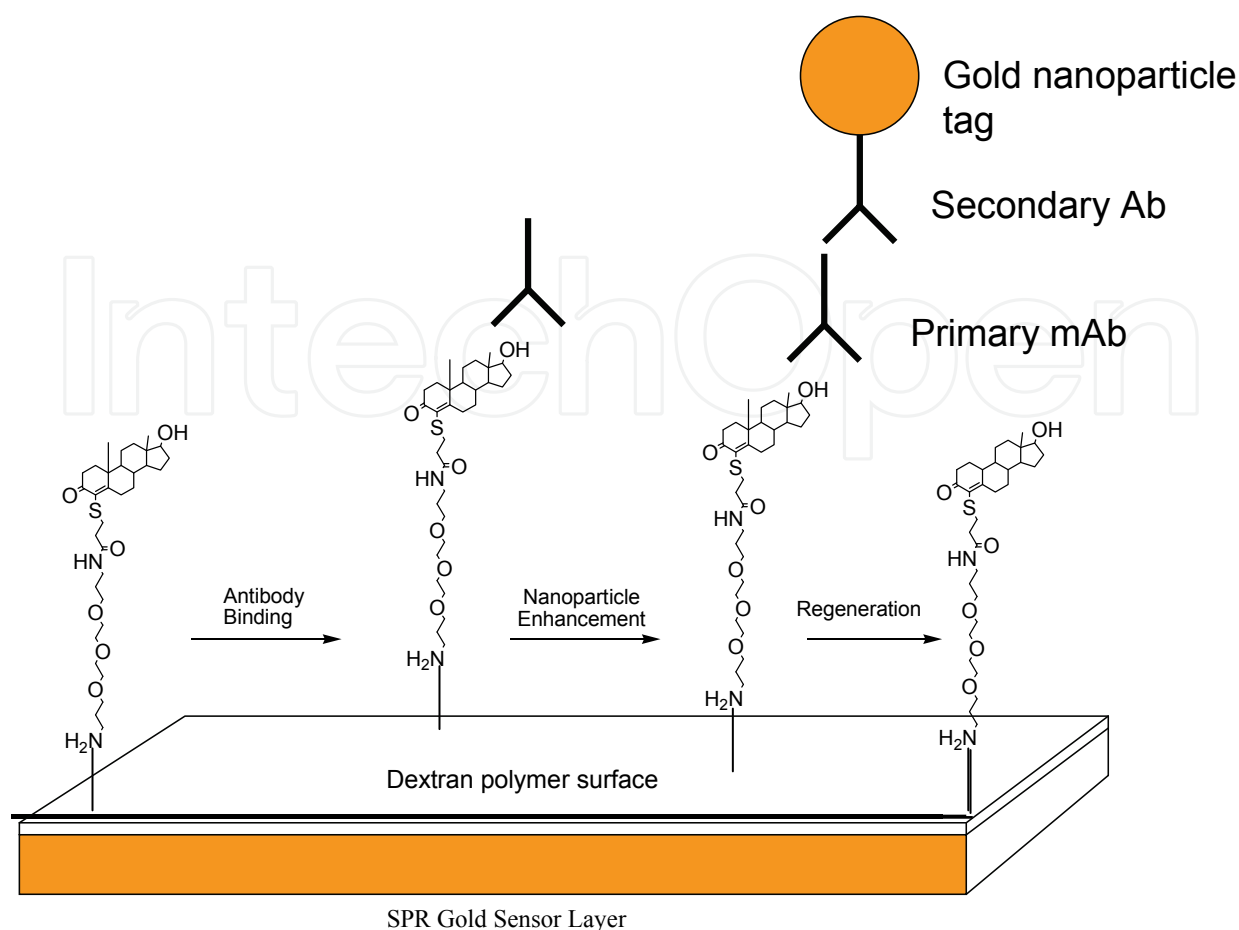


Fig. 3. Schematic showing the steroid immobilised SPR sensor surface, binding of primary antibody and labelling with secondary antibody – gold nanoparticle conjugate.

the binding interaction were applied. First, a secondary antibody that recognises the primary antibody, was bound in a separate binding step to add signal to the binding interaction and allow significant reductions in the concentration of the primary antibody (Fig. 3). LOD of 20.1 pg/mL could now be obtained for progesterone and signal was enhanced 8.1-fold (Mitchell et al., 2005) (Fig. 4).

As well as using secondary antibody there are other means of adding high mass tags to the binding interaction. Gold nanoparticles have a high mass contained in nanospheres the diameter of which can be altered as required between about 1 nm – 100 nm by altering the conditions of their synthesis. This size range is ideal for tagging antibodies and furthermore, gold nanoparticles can be readily conjugated to proteins such as antibodies through charge interactions. Gold nanoparticle enhancement has been applied to large molecule immunoassays (Gu et al., 1998; Lyon et al., 1998; White & Rosi, 2008) but significant steric challenges exist for its use with small molecules. Gold nanoparticles of 25, 45, 55 and 70 nm diameters were examined as tags for secondary antibodies for signal enhancement in small molecule SPR immunosensing. 25 nm nanoparticles were found to give the highest signal enhancement and were applied in a progesterone assay. Signal enhancement further increased to 13-fold and the LOD could be reduced to 8.6 pg/mL for progesterone (Fig. 5) (Mitchell et al., 2005). By application of gold-secondary antibody signal enhancement, the LOD could thus be reduced by more than two orders of magnitude (Mitchell et al., 2005),



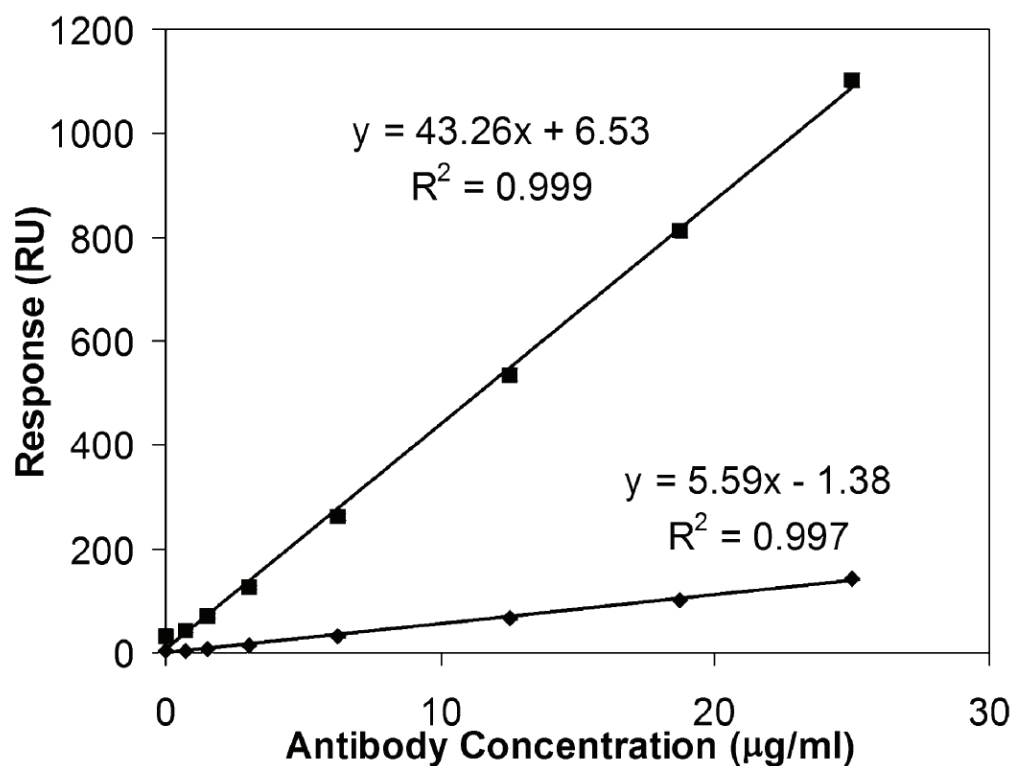


Fig. 4. Plots of SPR response (RU) vs. antibody concentration (µg/ mL) for unenhanced mAb only assay (◆) and secondary antibody enhanced assay (■). Reprinted from Analytical Biochemistry, 343, Mitchell, J. S.; Wu, Y.; Cook, C. J.; Main, L. Sensitivity enhancement of surface plasmon resonance biosensing of small molecules. 125-135, 2005, with permission from Elsevier.

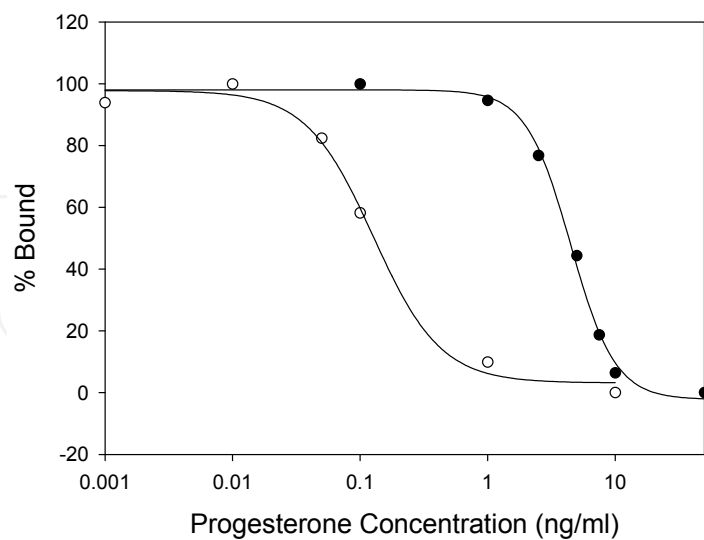


Fig. 5. Progesterone SPR immunoassay standard curves for assay without signal enhancement (●) and with gold nanoparticle signal enhancement (○), showing improvements in LOD. Reprinted from Analytical Biochemistry, 343, Mitchell, J. S.; Wu, Y.; Cook, C. J.; Main, L. Sensitivity enhancement of surface plasmon resonance biosensing of small molecules. 125-135, 2005, with permission from Elsevier. (RSC) ([www.rsc.org](http://www.rsc.org))

allowing sensitive detection of steroid hormones at the concentrations typically found in human saliva. The signal enhancement arising from the gold labelling could result not only from the added mass but also from cooperative plasmon coupling between the gold nanoparticles and the underlying gold sensor surface. This effect has been suggested before with large molecule binding targets (Lyon et al., 1999).

Using bulky nanoparticles to enhance SPR signal strength, adds to the potential for steric hindrance in immunoassays of small molecules. These assays were constructed around a “double spacer” system whereby spacers or linkers project the antigen out from the sensor surface and hold the primary antibody out from the gold surface, thus allowing space for the antigen / antibody interactions and ensuring optimal sensor signal. Non-specific binding from the conjugated gold nanoparticles could be reduced significantly by the addition of polyethylene glycol (PEG)-400 or PEG-4000 (Mitchell et al., 2005). By using a 25 nm gold nanoparticle, the risk of the colloid settling or aggregating over the course of the assay is minimised. Bovine serum albumin (BSA) could also be used to further reduce non-specific binding (Mitchell et al., 2005).

## 5. Small molecule analytes

The immunobiosensor format outlined in the previous sections is quite generic and can be applied to a wide range of small molecules provided they have a suitable site for conjugation and have high affinity monoclonal antibodies available (Fig. 6). The SPR immunobiosensor approach outlined was first tried with progesterone as a model compound. Progesterone has well developed monoclonal antibodies and its measurement is of significant interest in the monitoring of oestrous cycles in animals and in the early

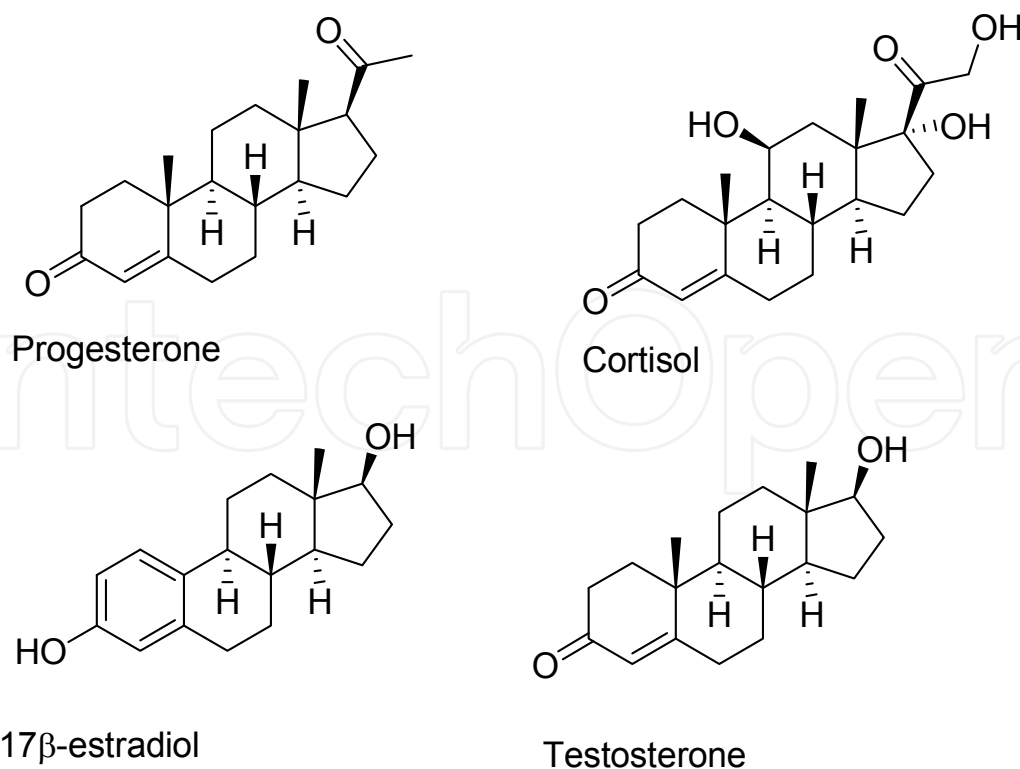


Fig. 6. Structures of steroid analytes covalently conjugated to SPR sensor surface through the 4-position



detected as could enhancement with gold-nanoparticle labelled secondary antibody (Fig. 8). The system could also be regenerated and had low non-specific binding from the gold nanoparticles (Sharpe et al., 2008).

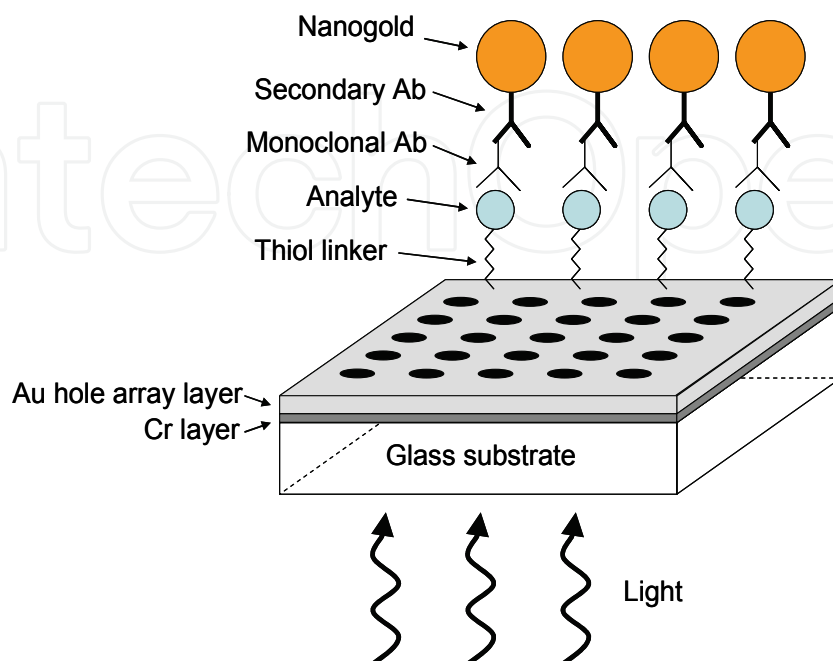


Fig. 8. Schematic showing a gold nanohole array functionalised with analyte (cortisol) conjugated to a thiol linker. The analyte is bound by a primary monoclonal antibody and then labelled with a secondary antibody-nanogold conjugate. Reprinted with permission from Sharpe, J. C.; Mitchell, J. S.; Lin, L.; Sedoglavich, N.; Blaikie, R. J. (2008) *Anal Chem*, 80, 6, 2244-2249. 2008 American Chemical Society.

## 6. Measurement in saliva

Human saliva contains very high concentrations of high molecular mass components such as mucins. These compounds can bind to SPR sensor surfaces and generate erroneous responses. This problem is particularly acute for small molecules, such as the steroid hormones, present at very low concentrations. Cortisol is present typically at 0.1 – 10 ng/mL in human saliva and testosterone at 29-290 pg/mL (Ellison et al., 2002). Measurement in saliva is particularly advantageous as it allows non-invasive detection of small molecules and regular sampling. Samples ideally need to be analyzed rapidly in near real-time, so chemical extraction and extensive pre-treatment of samples are not options.

Standards of cortisol and testosterone spiked into stripped human saliva could be used for the measurement of salivary hormonal concentrations. These standards could be passed through a BIAcore SPR instrument without clogging of the microfluidics (Mitchell et al., 2009). In the case of cortisol, saliva samples from human subjects were analyzed in the BIAcore without extensive pre-treatment through the addition of sodium dodecyl sulphate (SDS) to the antibody diluent at carefully controlled concentrations. The SDS is thought to minimise adhesion of salivary mucins to the sensor surface through charge repulsion, but at the concentrations used it did not affect the antibody or compromise the assay sensitivity greatly (Mitchell et al., 2009). The non-specific binding was reduced to a consistent and low

level enabling an assay curve to be formed without significant interference from high-molecular mass species (Mitchell et al., 2009). An LOD of 49 pg/mL was obtained for salivary cortisol, well below that required for analysis of saliva samples. Additionally, a strong signal sensitivity of 162 RU.mL/ng was attained allowing accurate discrimination of concentrations ranging to below 1 ng/mL (Mitchell et al., 2009). Despite the use of SDS, interassay CVs of 13.5% were achieved, comparable to many ELISA (Mitchell et al., 2009; Mitchell et al. 2004). No clogging of the microfluidics was observed despite the high viscosity of saliva. To ensure the accuracy of the salivary cortisol measurements, samples from 40 healthy male volunteers were compared to results from RIA. The slope of the correlation line was close to 1 with an  $r = 0.94$ , showing good agreement between the two techniques (Mitchell et al., 2009) (Fig. 9). Binding and regeneration steps could be completed in 10 min per sample thus allowing for a fast throughput of samples. Another advantage of this method is that pre-concentration of samples is avoided as the technique has sufficient sensitivity to measure samples without complex pre-treatment (Mitchell et al., 2009). Furthermore, measurement can be made in small sample volumes (50  $\mu$ L of sample is sufficient).

This SPR technique for measurement of salivary cortisol provides a useful means of monitoring hormone concentrations non-invasively in near-real-time and can be applied to both diagnostic and stress monitoring applications. As the technique is largely generic, it can be extended to other target small molecules found in human saliva. A later cortisol SPR study using a different sensor surface approach has failed to achieve comparably low LOD (Frasconi et al., 2009).

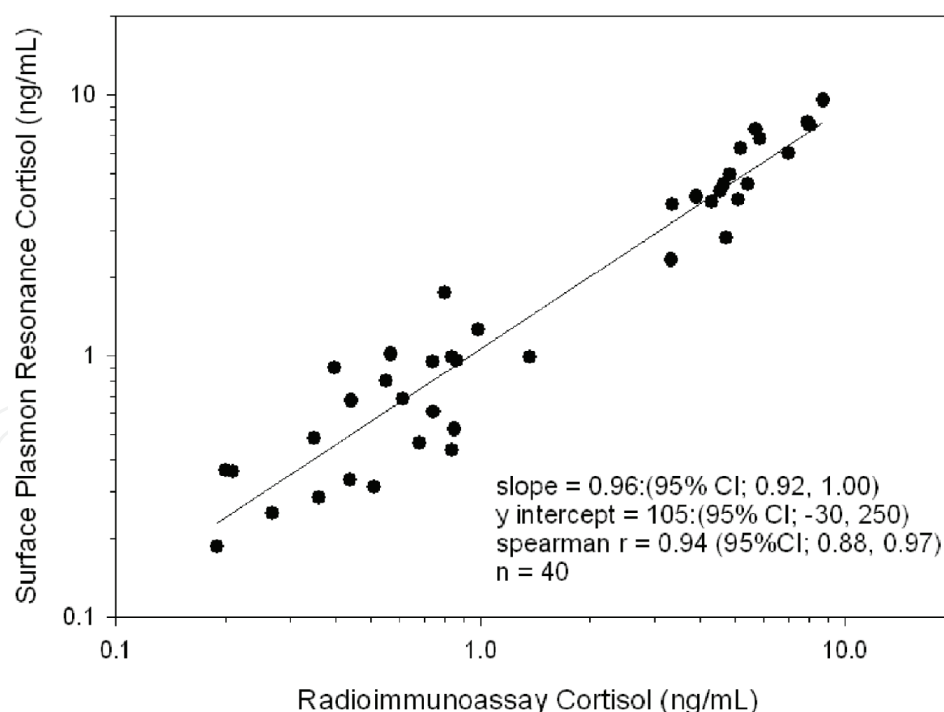


Fig. 9. Correlation plot comparing concentration determinations made by the SPR cortisol immunoassay and salivary cortisol radioimmunoassay. Rapid ultrasensitive measurement of salivary cortisol using nano-linker chemistry coupled with surface plasmon resonance detection. *Analyst*, 134, 2, 380-386 – Reproduced by permission of The Royal Society of Chemistry



## 7. Context in wider plasmonic biosensor research

The ever widening field of plasmonic biosensor research largely falls into three main areas. 1. The use of SPR to study biomolecular interactions. This includes antibody, oligonucleotide, receptor and enzyme research and involves using SPR as a tool to probe biological systems. 2. The study of plasmon fields and their relationship to the noble metal surfaces from which they emanate (Lyon et al., 1999). This typically involves using quantum, electrical and optical physics to systematically investigate the plasmon resonance phenomenon at a fundamental level. 3. The use of SPR biosensors to detect large molecule targets, including proteins and DNA (Huang et al., 2008). The use of SPR for the sensitive measurement of low concentrations of small molecules has not received as much attention as assays for proteins and oligonucleotides. There is now growing research interest in improving small molecule SPR immunoassay through design of the sensor interface (Mitchell et al., 2005; Miura et al., 2008; Shankaran et al., 2007). Recent studies have examined the use of SAM layers combined with protein conjugates to fabricate sensor layers for a herbicide (2,4-D, LOD of 10 pg/mL) (Kim et al., 2006), benzaldehyde (Gobi et al., 2007 b) and a steroid hormone (progesterone, LOD of 4.9 pg/mL) (Yuan et al., 2007) either with or without protein or nanogold enhancement. This approach has also been applied to measurement of the antimicrobial chloramphenicol (Yuan et al., 2008). Further work using direct covalent immobilisation to SAMs for TNT analysis has also been combined with secondary antibody signal enhancement (LOD of 50 pg/mL) (Mizuta et al., 2008) and a similar strategy developed for detection of benzaldehyde using PEG linker chains (Gobi et al., 2007 a). Immobilisation of histamine through a SAM layer without enhancement has yielded poorer LOD (3 ng/mL) (Li et al., 2006). Gold nanoparticle enhancement has also been used to improve LOD in the immunoassay of the metabolite estriol-16-glucuronide (LOD of 14 pg/mL) (Jiang et al., 2009) using protein conjugate immobilized on CM dextran. A protein conjugate to a small molecule dye has also been used as the high mass labelled antigen in a competitive SPR immunoassay with immobilised antibody (Aizawa et al., 2007) and biotin-BSA has been used as a high mass label for 2,4-D immunoassay (Kim et al., 2007). With gold nanoparticle enhancement of small molecule SPR immunoassays now well established, it is hoped that it will be successfully applied to an even broader range of compounds. The issue of biofouling in small molecule SPR is also receiving attention but often the techniques used involve total removal of the background matrix by time-consuming methods such as flow filtering where blockages can occur and LOD are too high (Stevens et al., 2008) or else extensive dilution of samples (Gillis et al., 2002) which worsens the practical LOD.

By combining surface and conjugation chemistry with immunology and optics, it is possible to create new sensor interfaces that offer significantly improved detection of small molecules, not only in buffer but also in real biological samples. The research detailed in this chapter represents several advances in the use of SPR to detect small molecules of biological significance at very low concentrations and particularly in a complex biological matrix.

## 8. Conclusions and future research

The sensitive measurement of very low concentrations of small molecule analytes is of great value in the diagnosis of disease, study of human and animal physiology and the monitoring of product quality and environmental integrity. Many small molecules are

routinely measured by traditional RIA or ELISA in expensive, time-consuming and centralised processes. In this chapter we have examined how SPR immunobiosensors can be used to measure very low small molecule concentrations accurately, quickly, affordably, with low labour requirements and even in complex biological matrices on highly re-usable sensor surfaces. The use of chemical linkers to covalently immobilise steroid antigens to the sensor surface allows the surface to be re-used many times even under harsh regeneration conditions. By selecting a conjugation position and linker length that optimises antigen presentation in the flow-through assay format, antibody binding can be maximised and high signal sensitivities obtained. The use of hydrophilic and non-immunogenic linkers also improves specific binding response. Secondary antibody labelling of the primary monoclonal antibody binding interaction is a convenient means of enhancing the sensor signal and further reducing LOD. Enhancement is further increased by the use of gold nanoparticles which add further mass and cooperatively enhance the plasmon shift. As the technology is generic, it can be applied to a wide range of small molecule targets. The application of this technology to the sensitive measurement of salivary cortisol has been demonstrated with the addition of SDS surfactant to minimise non-specific binding and allow measurement without chemical extraction or extensive pre-treatment of samples.

To achieve the next generation of SPR biosensors for small molecules, future research will likely focus on expanding the types of small molecule analyte conjugated to the sensor surface and on examining alternative gold substrate designs to further improve sensitivity. The covalent nanolinker approach described in this chapter has been applied to a range of steroid hormones but it is also amenable to use with other small molecules such as pesticides, pharmaceutical residues and human and animal metabolites. Given that new regulations increasingly require sensitive measurement of many of these compounds, there is a very real role for SPR biosensors to play in semi-automated, high-throughput analysis of environmental and food samples. Biomedical testing laboratories may increasingly look to semi-automated alternatives to traditional plate-based assay formats as their sample loads increase for diagnosis and monitoring of medical conditions. Accessing new classes of analytes is limited only by the conjugation technique used to attach the linker to a point distant from existing functional groups. Alternative gold substrates that are attracting much attention include the use of “nano-islands” of deposited noble metal. These islands can generate localised surface plasmons (LSPs) particularly around their vertices and can generate very steep plasmon field gradients which could potentially result in greater sensitivity. Another emerging technique is the use of gold nanohole arrays as described above. Here, optical field concentration around the nanoholes offers the potential for further improvements in sensitivity.

When developing biosensor technologies for highly sensitive detection of small molecules, it is crucial to consider the chemistry of the sensor / sample interface, as it is here that the critical binding interactions needed for measurement take place. By rationally designing this interface, one can greatly improve the performance of the biosensor by maximising specific binding interactions, minimising non-specific binding and biofouling and creating chemically stable and highly re-usable sensor interfaces. This approach combined with the rapid advances in plasmonic transduction technology, particularly plasmonic substrate design, offer great promise for even more sensitive and reliable small molecule biosensors.

## 9. Acknowledgments

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A biosensor is defined as a detecting device that combines a transducer with a biologically sensitive and selective component. When a specific target molecule interacts with the biological component, a signal is produced, at transducer level, proportional to the concentration of the substance. Therefore biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. Bringing together researchers from 11 different countries, this book covers a wide range of aspects and issues related to biosensor technology, such as biosensor applications in the fields of drug discovery, diagnostics and bacteria detection, optical biosensors, biotelemetry and algorithms applied to biosensing.

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